

Epitope mapping of the house-dust-mite allergen Der p 2 by means of site-directed mutagenesis

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Recombinant Der p 2, expressed in the baker's yeast *Saccharomyces cerevisiae*, was used as a tool to determine IgE- and monoclonal antibody (mAb)-binding sites on this allergen. For this purpose, mutant molecules were produced by application of site-directed mutagenesis. The amino-acid residues spanning cys21–cys27 and cys73–cys78 were deleted, thus preventing loop formation through disulfide bonds. Charged residues in three predicted antigenic sites (residues 45–48, 67+69, and 88–90) were replaced by alanine residues. IgE- and mAb reactivity to these mutants was compared to that to "wild type" Der p 2. Residues spanning cys73–cys78 were involved in the antigenic binding site for mAb α DpX. Mutations in the areas adjacent to this loop (i.e., 67+69 and 88–90) had similar effects on this mAb (10- to 20-fold decreases in reactivity were observed), supporting the suggestion that these areas are involved in this antigenic structure. The area of residues 45–48 was shown to be involved in an epitope for mAb 2B12. The reactivity of mAb 7A1 was influenced by substitutions of residues 45–48 as well as 88–90. Deletion of the residues spanning cys21–cys27 resulted in decreased reactivity to three mAbs (10E11, α DpX, and 7A1). From these observations, it may be concluded that binding of different mAbs is influenced by the same mutations and that the binding of single mAbs is influenced by two or more mutations scattered over the allergen molecule. These findings can point in two directions: minor amino-acid changes result in disruption of the overall conformation of the allergen, or distant sites are close together in the three-dimensional structure of the allergen. Decreased IgE reactivity was observed with all mutant molecules, varying between patients. The observed effects ranged from 5- to 1000-fold. Deletion of the amino-acid residues spanning cys21–cys27 and cys73–cys78 had the strongest effect on IgE reactivity, where decreases up to 1000-fold were observed. Such mutants might be useful tools to improve the safety of allergen-specific immunotherapy.

**G. A. J. Hakkaart^{1,2}, R. C. Aalberse¹,
R. van Ree¹**

¹Central Laboratory of The Netherlands Red Cross Blood Transfusion Service and the Laboratory for Experimental and Clinical Immunology of the University of Amsterdam, Amsterdam; ²Section for Molecular Biology, Department of Molecular Cell Biology, Biocentrum Amsterdam, University of Amsterdam, Amsterdam, The Netherlands

Key words: allergen; antigenic binding sites; baker's yeast; house-dust mite; IgE; monoclonal antibodies; recombinant Der p 2; *Saccharomyces cerevisiae*; site-directed mutagenesis.

R. van Ree
Central Laboratory of The Netherlands Red Cross Blood Transfusion Service and the Laboratory for Experimental and Clinical Immunology of the University of Amsterdam
Plesmanlaan 125
1066 CX Amsterdam
The Netherlands

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The house-dust-mite allergen Der p 2 is a protein of 129 amino-acid residues. The cDNA was originally isolated by Chua et al. (1). Der p 2 contains six cysteine residues which form three disulfide bridges (2), resulting in two small loops and a close link between the N- and the C-terminus.

Epitope mapping of major allergens is a first step in the development of safer immunotherapeutic allergen preparations with low IgE-binding capacity. Several attempts have been made to map epitopes on Der p 2. Human IgE did not show reactivity to peptides (20–30 amino-acid residues)

expressed in *E. coli* from a random fragment Der p 2 cDNA library, and recombinant peptides comprising half the linear sequence showed only weak binding to IgE antibodies (3). Another approach was performed in our laboratory by

Abbreviations. α -gal: α -galactosidase; mAb: monoclonal antibody; PBS: phosphate-buffered saline; PBS-T: PBS+0.1% (v/v) Tween-20; PBS-AT: PBS-T+0.3% (w/v) BSA; RAST: radio-allergosorbent test; rec: recombinant; RU: RAST unit; WT: wild type.

means of synthetic peptides (4). Whereas this method was demonstrated to be an effective tool in epitope-mapping studies on various allergens, such as Amb a 3 (5), Chi t 1 (6), Gad c 1 (7), Fel d 1 (8), Cor a 1, and Bet v 1 (9), it was less useful for Der p 2. Very few patients had appreciable amounts of IgE against only one peptide (residues 65–78) from a panel of overlapping peptides, spanning the complete Der p 2 molecule (4). Furthermore, from a panel of Der p 2-specific mAbs, none showed reactivity to these peptides (van 't Hof, personal communication). These experimental data agree with the finding that the B-cell epitopes on the Der p 2 allergen are destroyed upon reduction and alkylation (10). Therefore, it is likely that the B-cell epitopes on the group 2 allergen are highly dependent on its conformation, making these techniques for epitope mapping less efficient.

Our approach to investigate B-cell epitopes of Der p 2 was to construct mutants of a yeast-derived recombinant Der p 2 by means of site-directed mutagenesis. The nonmutated, or "wild-type" (WT), Der p 2 has been shown to be almost indistinguishable from natural Der p 2 (11). In two recent reports with a similar approach, the integrity of the three disulfide bridges has been shown to be of importance for IgE and monoclonal antibody (mAb) binding (12, 13). In the present study, the two small cysteine loops were deleted. In addition, several mutations were introduced in potential antigenic sites, selected on the basis of hydrophilicity and surface probability in combination with secondary-structure and backbone-flexibility predictions (14–20).

Herein we describe the production and isolation of these Der p 2 variants from yeast and their reactivity to IgE and mAbs compared to the non-mutated Der p 2.

Material and methods

Sera

Sera ($n=20$) were collected from subjects showing IgE reactivity (RAST units >0.3) to Der p 2.

Monoclonal antibodies

The anti-Der p 2 mAbs used were α DpX (21), 10E11 obtained after immunization of BALB/b mice with a fraction from mite whole-body extract <60 kDa (van Leeuwen & Aalberse, unpublished), 2B12B3 (22), and 7A1 (23). For application in RAST, ascites or culture supernatants were used. mAbs 10E11 (culture supernatant) and α DpX (ascites) were also protein A purified. α DpX was bioinylated, 0.8 mg

NHS-LC-biotin (Pierce, Rockford, IL, USA)/1.2 mg mAb.

Molecular cloning

Molecular cloning of Der p 2 was performed as described elsewhere (11). The Der p 2-containing plasmid pUC-p2 (1) was kindly provided by Dr W. R. Thomas, Subiaco, Australia.

Mutagenesis of the Der p 2 cDNA

Mutations of the Der p 2 cDNA were generated by means of pSELECTTM (Promega, Madison, WI, USA) with a few modifications. The 594 bp *Eco* R I fragment from pUC9-p2 was isolated and ligated into pSELECT, resulting in pSELECT-p2. The single-stranded DNA was isolated by means of infecting the pSELECT-p2 transformant with helper phage M13K07. The *in vitro* mutagenesis was carried out as described in the manual. The putative mutant pSELECT-p2 DNA was isolated from *E. coli* BMH 71-18mutS and hence transformed to *E. coli* JM 110. The transformants were not plated out, but grown in 250 ml 2 YT with ampicillin, and hence the plasmid DNA, was isolated. From this DNA, the 520 bp *Bcl* I–*Hin* d III fragment was isolated. This fragment was ligated into the expression vector pSAY1 (11) digested with *Bcl* I and *Hin* d III. These pSAY1-p2 constructs were then screened by sequence analysis. The oligonucleotides used in this procedure, and the resulting amino-acid changes are listed in Table 1.

Production of recombinant Der p 2 allergens from yeast

Yeast strain *Saccharomyces cerevisiae* BJ 1991 (*MAT α* , *leu2-1*, *trp1-1*, *ura3-52*, *prb1-1122*, *pep4-3*) (24) was transformed (25) with the appropriate plasmids, and transformants were selected on minimal plates (0.67% yeast nitrogen base without amino acids, 2% glucose, 20 μ g/ml uracil, 20 μ g/ml tryptophane, and 2.2% agar). The transformants were grown for 40 h in minimal medium; subsequently, the yeast culture was diluted in a 10-fold of this volume in minimal medium with galactose instead of glucose, all at 30°C. After 1 week of growth, the cells were spun down, and the growth medium was further analyzed.

Purification and quantification of Der p 2

A volume of 500 ml growth culture – containing WT rec Der p 2 – was concentrated on an Amicon ultrafiltration cell over a diaflo Ultrafiltration

Table 1. Oligonucleotides used for mutagenesis of Der p 2. Sequences of oligonucleotides used for mutagenesis of Der p 2 cDNA are given with their resulting substitutions in protein. Mutated nucleotides are depicted in both italic and lower case. Deletions in cDNA are represented as –

	Oligonucleotide	Mutation in protein
M45–48	GCCGTTTTCGAAGCCAACgcAgcCgCAg cAACGGCTAAAATTGAAATCAAAGCC	gln45.asn46.thr47.lys48 Ø ala.ala.ala.ala
M67+69	GAAGTTGATGTTCCCGcTATCGcTCCAA ATGCATGCCATTACATG	gly67 Ø ala; asp69 Ø ala
M73–78	GGTATCGATCCAAATGCAgc– cgcCCCATTGTAAAGG ACAACAATATG	cys73 Ø ala, cys78 Ø ala; Δ 74–77
M88–90	GGACAACAATATGATgcTgcAgcTACAT GGAATGTTCCG	ile88.lys89.tyr90 Ø ala.ala.ala
M22–26	CAAAAAAGTTTTGGTACCAGGATGC– TGTATCATTCAT CGTGGTAAACC	Δ 22–26

membrane YM10 (Amicon Corp, Danvers, MA, USA) to a volume of 10 ml. A volume of 1 ml of concentrate was applied to a Superdex 75 HR10/30 column (Pharmacia Biotech, Uppsala, Sweden), equilibrated in 0.05 M NaPO₄/0.5 M NaCl/pH 7. The bed volume was 23 ml, and the flow rate was 0.5 ml/min. Der p 2 eluted at between 29 and 36 min (14.5 and 18 ml), resulting in a purity >95%, as judged by SDS–PAGE/silver staining (not shown). The protein concentration of this purified allergen was measured with BCA protein assay reagent (Pierce, Rockford, IL, USA) at 37°C, according to the manufacturer's instructions. BSA was used as protein standard. From this purified Der p 2, a standard was prepared in ×50 concentrated pSY1 growth culture, containing 106 µg Der p 2/ml. This standard plus three consecutive threefold dilutions, together with samples of concentrated growth media containing mutant Der p 2, were electrophoresed on an ExcelGel™ SDS gradient 8–18 (Pharmacia Biotech, Uppsala, Sweden) and silver-stained according to the manual of the producer. The intensities of the Der p 2 bands were scanned with MacImage™ (Xerox Imaging Systems, Inc., Sunnyvale, CA, USA). From the Der p 2 standard curve, the concentration of the allergen mutants in culture supernatants was calculated by Slide Write Plus 6.0.

Der p 2 two-site ELISA

A two-site ELISA for Der p 2 was performed as described previously (11). For quantification purposes, standards were prepared from purified recombinant Der p 2 with known protein content (BCA, Pierce).

Preparation of the solid-phase antigen, RAST, and RAST inhibition

Concentrated (×50) growth medium (300–500 µl) containing 50 µg allergen was coupled to 300 mg

CNBr-activated Sepharose-4B (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's manual. The Sepharose was suspended in 50 ml of PBS/0.3% BSA/0.1% Tween-20 (PBS-T). According to the same procedure, 500 µl concentrated (×50) pSY1 growth medium was coupled to 300 mg Sepharose. The pSY1 growth culture was used as a negative control.

For the RAST, 500 µl (1 mg) Sepharose was incubated with 50 µl antibody sample overnight, end over end, at room temperature. After washing five times with PBS-T, an ¹²⁵I-radiolabeled antibody against mouse IgG (M1482, CLB) or human IgE (SH25-1-P7, CLB; affinity-purified before iodination) was added. After incubation overnight, end over end at room temperature, the Sepharose was washed four times with PBS-T and the gamma radiation was counted. Results were expressed as percent binding of total counts added.

For RAST inhibition, 50 µl of patient serum or mAb (diluted when necessary, to achieve nonsaturating conditions) was preincubated with 50 µl inhibitor. After 2 h of incubation at room temperature, 500 µl of Sepharose was added, and the RAST was further carried out as described above. The relative inhibitory potency (i.p.) of a particular mutant is defined as the ratio of the amounts of allergens required (rec WT Der p 2/mutant) for 50% inhibition of the RAST.

Results

Mutagenesis of Der p 2 cDNA

The efficiency of the mutagenesis was between 20% and 100% mutant constructs (four to 10 clones were analyzed for each mutation). Loops between cysteine residues 21–27 and 73–78 were deleted (Fig. 1: M22–26 and M73–78), and charged amino-acid residues in three regions (Fig. 1: M45–48, M67+69 and M88–90) were replaced by alanine residues.

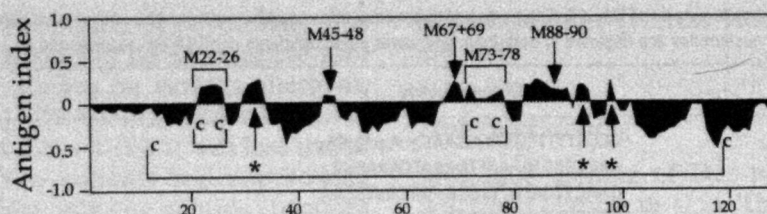


Fig. 1. Antigenicity plot of Der p 2 was derived from mature Der p 2 sequence (Chua et al. [1]) by means of MacVector™ (IBI-A Kodak, New Haven, CT, USA). Codes of mutants are represented. Substitutions and deletions are listed in Table 1. Asterisks refer to three other mutations; namely, arg31.gly32.lys33Øala.ala.ala, pro99Øala, and asn93Øala. Cysteine residues are indicated (c) as well as disulfide bonds between them.

In addition, charged residues in three other regions were replaced by alanine residues (namely, arg31.gly32.lys33Øala.ala.ala, pro99Øala, and asn93Øala, represented in Fig. 1 with asterisks). Since these substitutions did not result in a significant decrease of antibody binding in our hands, they are not discussed below.

Expression and quantification of the allergens

The (mutant) Der p 2 constructs were all transformed to yeast, and (mutant) allergen was harvested. Concentrated allergen-containing growth media were separated on SDS-PAGE, followed by silver staining (not shown). The intensities of the rec WT Der p 2 standard and rec WT Der p 2 growth culture were scanned. By this procedure, the concentration of Der p 2 in concentrated growth culture was calculated by linear regression: 140 µg/ml. This value was in good agreement with quantification in a two-site ELISA (150 µg/ml). The growth culture of all Der p 2 variants was also scanned, and the concentrations were calculated similarly (Table 2).

Table 2. Expression of Der p 2 and Der p 2 mutants. Scanned areas from Der p 2 standard, and allergens in culture supernatants are given. From plot of standard curve, concentrations were calculated. Dilution of allergen sample is represented.

Sample	Scanned area	Dilution	Concentration µg/ml
Der p 2	714539	1	106
Standard	524291	1	35
	377497	1	12
	304622	1	4
Der p 2	593911	2	140
M45-48	561404	1	61
M67+69	602394	1	77
M73-78	469320	2	66
M22-26	314853	1	6
M88-90	582888	2	138
α-gal	208286	1	—

Reactivity to mAbs

The binding to the five Der p 2 mutants was studied with four different mAbs in RAST (Table 3). In addition, RAST inhibitions were performed with mAbs 2B12, αDpX, and 10E11 and mutants M45-48, M67+69, and M73-78 as inhibitor (Fig. 2 and Table 4).

2B12B3

Substitutions in the area 45-48 (M45-48) resulted in a decrease in reactivity of approx. 300-fold with mAb 2B12B3, as judged by RAST inhibition. Deletion of the stretch of residues at 73-78 (M73-78) had a minor effect on the binding of this mAb: approx. fivefold reduction (Fig. 2A). The other three mutants studied did not show significant changes in binding to mAb 2B12B3.

αDpX

Substitution of residues 67 and 69 (M67+69) or deletion of the adjacent stretch of residues 73-78 (M73-78) had similar effects on the reactivity of mAb αDpX in RAST inhibitions, indicating that the area at residues 65-80 is crucial for the integrity

Table 3. Reactivity of Der p 2 mutants to different mAbs. Der p 2 RAST for four mAbs was carried out with Der p 2 and Der p 2 mutants. Sepharose was diluted five times in glycine Sepharose in PBS-AT. Dilution of mAbs were 7A1 40 000 (ascites); 2B12B3 81 000; and 10E11 20 and αDpX 10 000 (culture supernatants) times. Results are expressed as percentages of added counts

	2B12	αDpX	7A1	10E11
α-gal	0.4	0.8	0.5	0.4
Der p 2	43.2	47.3	18.1	37
M45-48	6.3	13.5	4.0	10.9
M73-78	26.9	9.3	20.3	6.4
M88-90	45.2	12.8	10.7	40.2
M22-26*	42.2	6.0	1.9	1.5
M67+69	42.9	4.3	19.6	25.9

*For M22-26, due to low expression level, 30 µg, instead of 50 µg, was coupled.

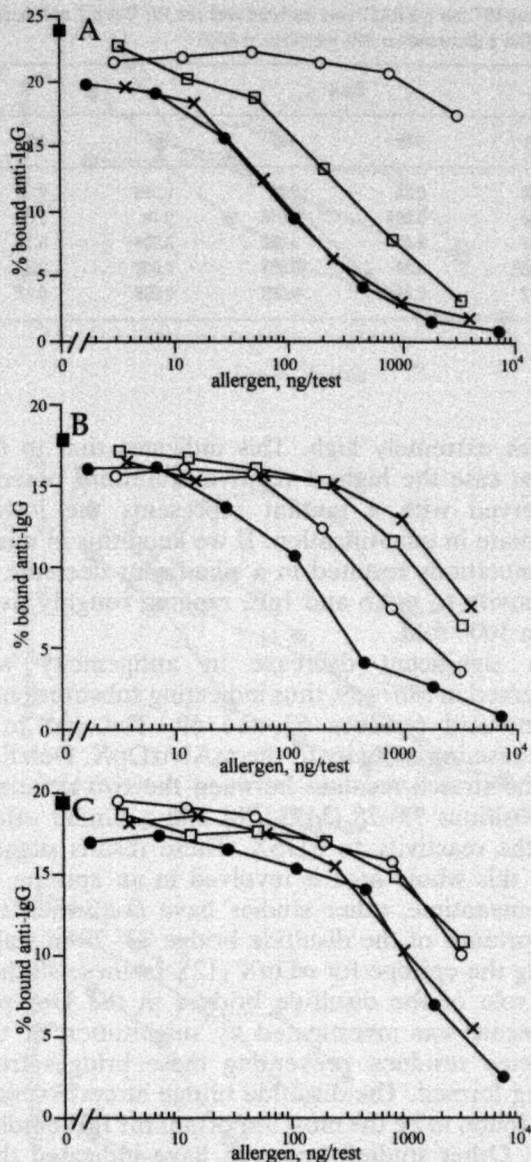


Fig. 2. RAST inhibition for mAbs. RAST inhibitions were carried out with (rec) Der p 2 on Sepharose. RASTs were inhibited with (rec) Der p 2 and Der p 2 mutants. Results are represented as percentage of added counts. Uninhibited RAST values are indicated by ■. A) mAb 2B12B3; B) mAb αDpX; C) mAb 10E11. ●=Der p 2, ○=M45-48, X=M67+69, □=M73-78.

of the epitope for αDpX. Mutants M67+69 and M73-78 were shown to be approx. 30- and 10-fold weaker inhibitors of the WT Der p 2 RAST, respectively (Fig. 2B). Even replacement of three charged residues a little further downstream of the C terminus (M88-90) still affected the binding of this mAb (Table 3). M45-48 had a minor effect – approx. three times weaker inhibitor – although it is located far from the area around the disulfide bond cys73-78 (Fig. 2B). However, it cannot be excluded that this effect falls within the

margins of accuracy of the protein-quantification method for the mutants. Finally, the deletion of amino-acid residues 22-26 (M22-26) resulted in significantly decreased binding of mAb αDpX (Table 3).

7A1

Mutations in the areas 45-48 (M45-48) and 88-90 (M88-90) had a significant effect on reactivity to mAb 7A1 (Table 3). M22-26 was no longer recognized by mAb 7A1. The mutants M73-48 and M67+69 did not show altered reactivity.

10E11

A previous study showed that addition of one alanine residue to the N-terminus had a drastic effect on the binding of mAb 10E11 (Hakkaart et al., paper submitted). The RAST data obtained in the present study indicate that mutants M67+69, M73-78, M45-48, and M22-26 show decreased reactivity to this mAb. In RAST-inhibition assays, no difference between WT Der p 2 and M67+69 was observed (Fig. 2C). For M45-48 and M73-78, inhibition curves shifted by a factor of 3. Therefore, as for mAbs αDpX and M88-90, it cannot be excluded that these differences are explained by inaccuracy in quantification of the mutants.

Reactivity to human IgE

Direct RAST. For 20 sera, RASTs were carried out with rec WT Der p 2 and mutants on Sepharose. At saturating solid-phase allergen concentrations, the ratios of IgE binding (mutant/WT) were in most cases >0.4. For M45-48, there were three exceptions (0.39, 0.22, and 0.36); for M73-78, two (0.36 and 0.38). Only for M22-26 did most sera (15/20) demonstrate lower IgE binding.

RAST inhibitions. For six of these sera, RAST inhibitions were carried out. For a selection of these experiments, inhibition curves are shown in Fig. 3A-C. The inhibitory potencies of the mutants, relative to WT Der p 2, are shown in Table 4 for all six sera analyzed.

M45-48: *gln45.asn46.thr47.lys48* *Qala.ala.ala.ala.* When charged residues at positions 45-48 were replaced by alanines, decreases in inhibitory potency varied from 3 to 100 times (Table 4). In Fig. 3A, inhibition curves are shown for serum 46 and serum CV. Approximately 30 times more of mutant M45-48 was needed for inhibition in the case of the latter serum.

Table 4. Relative inhibitory potencies of Der p 2 mutants. For three mAbs and six sera, rec WT Der p 2 RAST was inhibited with rec WT Der p 2 and mutants. Inhibitory potency for mutant is given as ratio of amounts of allergen required (rec WT Der p 2/mutant) for 50% inhibition of RAST

	Monoclonal antibodies				Sera				
	2B12B3	α DpX	10E11	2	46	135	136	CV	MV
M45-48	0.03	0.33	0.34	0.21	0.29	0.34	0.20	0.009	0.17
M67+69	0.90	0.057	0.44	0.072	0.88	0.004	0.018	0.18	0.91
M73-78	0.20	0.11	0.30	0.10	0.57	0.17	0.080	0.009	0.12
M22-26	Nt	Nt	Nt	0.001	0.002	0.14	0.007	0.002	0.07
M88-90	Nt	Nt	Nt	0.015	0.17	0.28	0.080	0.039	0.13

Nt: not tested.

M67+69: gly67 $\emptyset$ ala; asp69 $\emptyset$ ala. Two out of six sera tested in RAST inhibition showed an IgE reactivity of M67+69, comparable to that of Der p 2 (serum 46 and MV). For the other four sera, the decrease in reactivity was 5- to 250-fold (Table 4). Inhibition curves for a nondiscriminating serum (MV) and for a serum with a 250-times decreased reactivity (serum 135) are shown in Fig. 3B.

M73-78: cys73 $\emptyset$ ala; cys78 $\emptyset$ ala; Δ 74-77. In mutant M73-78, the cysteine loop at this position was deleted. This deletion resulted in decreases of reactivity of up to 100-fold (Table 4) observed for serum CV. IgE binding of serum 46 was hardly affected.

M22-26: Δ 22-26. In mutant M22-26, the amino acids between cys21 and cys27 were deleted, avoiding formation of the disulfide bridge between the cysteines. In the RAST inhibitions for the six sera tested, decreases in IgE reactivity ranged from 10- to 1000-fold (Table 4). Two extremes (serum 2 and 135) are shown in Fig. 3C.

M88-90: ile88.lys89.tyr90 $\emptyset$ ala.ala.ala. Replacing amino acids 88-90 by alanines resulted in 3- and 65-fold decreases in IgE reactivity (Table 4). IgE binding of serum 2 was most affected.

Discussion

In this study, rec Der p 2 mutants and rec "WT" Der p 2 were compared with regard to their reactivity to mAbs and IgE in order to identify antigenic binding sites.

A problem in this study was the quantification of each mutant in yeast culture supernatants. Quantification is ideally carried out with mono-specific antibodies. However, these antibodies behave differently with each mutant. Therefore, quantification was performed by an electrophoretic method (SDS-PAGE) in conjunction with silver staining and scanning. The accuracy of this method

is not extremely high. This indicates that in the worst case the highest relative inhibitory potency observed with a mutant represents the lowest estimate in quantification. If we keep this in mind, all mutations resulted in a significant decrease in reactivity to mAb and IgE, ranging roughly from 5- to 1000-fold.

A significant decrease in antigenicity was observed in M67+69, thus indicating substitution of amino-acid residues 67 and 69. This led to a decrease in reactivity to the mAb α DpX. Deletion of the stretch residues between the two cysteines at positions 73-78 (M73-78) had a similar effect on the reactivity to α DpX. These results suggest that this whole area is involved in an epitope. In the meantime, other studies have confirmed the importance of the disulfide bridge 73-78 in stabilizing the epitope for α DpX (12). In these studies, the role of the disulfide bridges in the Der p 2 molecule was investigated by substitution of the cysteine residues, preventing these bridges from being formed. The disulfide bridge at cys73-cys78 was found to be the most important for IgE binding (12). Other studies, however, have indicated that cys8-cys119 is the most important disulfide bridge in antigen binding (13, 26). In contrast, the first study reported this bridge to be the smallest contributor to antigenicity (12). The discrepancies may be caused by differences in the recombinant Der p 2 (or Der f 2) molecules tested, or may be related to differences in the sera used for the studies.

In our study, however, the largest decrease in IgE reactivity was observed in mutant M22-26; this was caused by the deletion of the stretch of amino-acid residues at cys21-cys27. It cannot be excluded that these amino-acid residues themselves are involved in an antigenic binding site, and that therefore the breaking of the disulfide bridge itself is not enough to cause a large decrease in antigenicity.

Another location found to be involved in an antigenic binding site was the stretch of residues 45-48. Replacement of these residues (M45-48) by alanine residues led to a decrease in reactivity

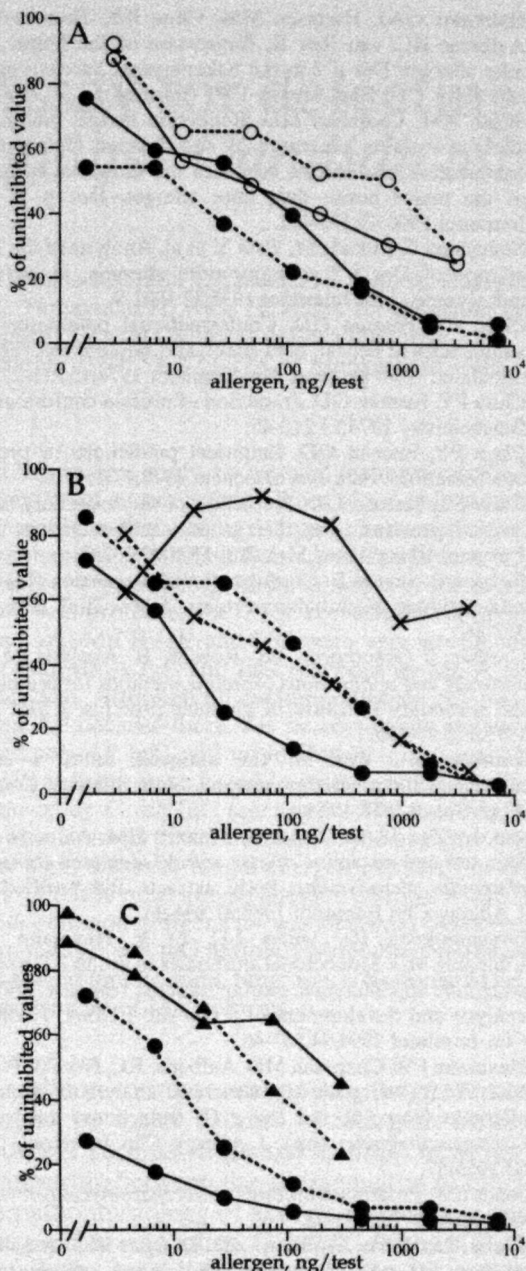


Fig. 3. RAST inhibition for IgE. RAST inhibitions were carried out with rec WT Der p 2 on Sepharose. RASTs were inhibited with rec WT Der p 2 and Der p 2 mutants. Results are represented as percentage of uninhibited value. ●=Der p 2, ○=M45-48, X=M67+69, △=M22-26. A) serum 46 (solid line) and serum CV (dashed line) with M45-48; B) serum 135 (solid line) and serum MV (dashed line) with M67+69; C) serum 2 (solid line) and serum 135 (dashed line) with M22-26.

to mAbs 2B12B3 and 7A1. The effect for 2B12B3 was the strongest, approx. a 300-fold decrease in reactivity. In IgE reactivity, a significant decrease could also be demonstrated.

The latter mutation together with mutation M88-90 resulted in a decrease in reactivity to mAb

7A1. The fact that both distance mutations influence the epitope for one mAb may suggest that the peptide sequences are somehow adjacent in the native molecule. This is supported by the finding that M45-48 also influences the reactivity to mAb α DpX. This mAb was shown to be directed to the area around disulfide bridge 73-78 (M67+69/M73-78/M88-90).

Deletion of residues at cys21-cys27 (M22-26) led to a large decrease in antigenicity. For three mAbs, decreased reactivity was observed. However, one mAb (2B12B3) showed strong binding. This might suggest that this mAb shows strong reactivity to partially unfolded Der p 2. The fact that this mAb shows strong reactivity to most of the mutants (except M45-48) points in the same direction. For M22-26, the decrease in IgE reactivity for sera tested was the largest; a decrease in relative inhibitory potency varying from 10- to 1000-fold was observed. The observation that all mAbs - except 2B12B3 - showed a large decrease in binding to M22-26 suggests that the overall conformation is disturbed in this mutant.

Although this approach resulted in the development of a set of Der p 2 variants with diminished antibody binding, some critical comments also have to be made on the antigenicity-prediction method. Amino-acid residues 31-33, 93, and 99 were also replaced by alanine. These variants did not show loss of antigenicity (results not shown), despite the fact that they were predicted. However, the computer predictions achieved with MacVector™ were a useful key in this epitope-mapping study. Random mutagenesis - followed by an effective screening procedure - might provide valuable additional information.

In conclusion, we constructed a set of Der p 2 variants with diminished immunologic reactivity. Preliminary studies indicated that these mutants also possess a decreased ability to induce histamine release from basophilic leukocytes (results not shown). Furthermore, these variants will be applicable in studies on the molecular mechanisms of mast-cell activation. Hence, monospecific IgE can be isolated by means of absorption IgE sera with particular Der p 2 mutant molecules. *In vitro* sensitization of basophilic leukocytes with this IgE (and combinations) may be used to study the minimal requirements needed to trigger the basophilic leukocytes (or mast cells). Furthermore, since all variants (except M22-26) tested still possessed the ability to induce histamine release from basophilic leukocytes, double or triple mutants might be developed from these experimental data, in order to obtain variants that lack biologic activity. Some of these variants may be useful in immunotherapy.

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